STUDIES ON THE ACTION OF PENICILLINASE

EXPERIMENTAL WOUND INFECTIONS AND EXPERIMENTAL IMMUNIZATION TO PENICILLINASE IN RABBITS

by

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EXPERIMENTAL WOUND INFECTIONS AND EXPERIMENTAL IMMUNIZATION TO PENICILLINASE IN RABBITS

AKADEMISK AVHANDLING

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This thesis is based on evidence presented in the following articles:

I. Tacking, Rolf: THE EFFECT OF PENICILLINASE-PRODUCING BACTERIA ON THE TREATMENT OF MIXED INFECTIONS IN RABBITS WITH PENICILLIN (79).[1]

II. —— PENICILLINASE-PRODUCING BACTERIA IN MIXED INFECTIONS IN RABBITS TREATED WITH PENICILLIN (80).

III. —— PENICILLINASE-PRODUCING AND PENICILLIN-SENSITIVE BACTERIA IN TWO SEPARATE SITES OF INFECTION IN RABBITS TREATED WITH PENICILLIN (81).

IV. —— IMMUNIZATION EXPERIMENTS WITH PENICILLINASE (82).

In this thesis the articles are referred to in the Roman numerals given above.

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[1] These articles are published in Acta Pathol. et Microbiol. Scand. (see references 79, 80, 81 and 82).

INTRODUCTION

Penicillinase, an enzyme which in the presence of certain bacteria is capable of inhibiting the antibiotic activity of penicillin, was first observed by Abraham and Chain (1). They found that a strain of *Escherichia coli* growing freely in the presence of considerable concentrations of penicillin produced no appreciable reduction in the antibacterial effect of the penicillin, but that an extract of the same organism made with Booth and Green's bacterial crushing mill (11) quickly inactivated penicillin at 37° C.

Penicillinase is of protein nature. It cannot be dialysed through cellophan, is destroyed by heating at 90° C for 5 minutes, and is adsorbed onto Seitz filter.

It exists in two forms, intracellular and extracellular. Intracellular penicillinase is bound within the bacterial bodies, and may be liberated from old cultures by autolysis of the bacteria. Extracellular penicillinase passes more or less completely into the surrounding medium.

Production of penicillinase in the presence of penicillin has been demonstrated in the following gram-positive and gram-negative bacteria: *Bacillus subtilis mesentericus group* (9, 23, 24, 87, 91), *Bacillus anthracis* (8, 9, 25, 60), *Staphylococci* (1, 3, 10, 36, 42, 48, 49, 61, 74, 76, 91 etc.), *Micrococcus lysodeicticus* (1, 91), *Mycobacterium tuberculosis* (91, 45), *Pasteurella* (91), *Escherichia coli* (66, 91), *Paracolobactrum* (9, 12, 41, 69, 78), *Aerobacter aerogenes* (5, 9, 50, 78), *Klebsiella pneumoniae* (56), and *Shigella-* (9), *Salmonella-* (9, 42), and *Proteus* (42, 53, 66) bacteria.

The action of penicillinase on penicillin in vitro has been examined by various authors (4, 30), who have found that a new acid group is formed during the reaction. Penicillinase was considered to be a special type of peptidase.

Perlstein and Liebmann (67, 68) have shown that penicillinase is antigenic. They produced an antipenicillinase-immune serum by injecting penicillinase intravenously in rabbits, which gives precipitation with penicillinase. Further, they demonstrated that this antipenicillinase-immune serum protected penicillin from destruction by penicillinase in vitro.

Housewright and Henry (42, 43), too, produced an antipenicillinase-immune rabbit serum, and demonstrated in vitro that this serum through inhibition of penicillinase increased the penicillin sensitivity of bacteria producing extracellular penicillinase. In addition, they showed that 90

per cent of the effect of penicillinase was inhibited when the enzyme was left in contact with antipenicillinase-immune serum at 37° C for one hour, and that inhibition commenced immediately.

The action of penicillinase in vivo has been studied by Levaditi, Vaisman, and Pérault (57, 58). They inoculated mice intraperitoneally with *Staphylococcus aureus,* and found that a subcutaneous injection of penicillinase produced by *Escherichia coli* was capable of inhibiting the action of penicillin injected subcutaneously at another site.

PRESENT INVESTIGATIONS

As indicated in the introductory paragraphs, the effect and mode of action of penicillinase have been studied elaborately in vitro, whereas only a few experiments have been carried out in vivo.

On the basis of clinical material it has been suggested and later confirmed that it is not possible to affect penicillin-sensitive bacteria by penicillin in the presence of pencillinase-producing bacteria (6, 15, 18, 39, 40, 51, 86).

The fact that infections due to penicillinase-producing bacteria are extremely common in hospital practice (22, 52, 54, 59, 71, 88, etc.) makes it of prime importance to clear up the problem of the interfering action of penicillinase on penicillin treatment. In the investigations to be described rabbits were used as test animals, wound-infections being chosen as the most suitable kind of infection.

This investigation is concerned with the importance of penicillinase in vivo. Thus none of the other enzymes or toxins produced by bacteria has been subjected to study.

The action of penicillinase was studied in wounds where penicillin-sensitive bacteria occurred in the presence of bacteria producing penicillinase intra- or extracellularly.

Experiments were carried out on wounds infected with penicillin-sensitive organisms in animals with a simultaneous infection due to a penicillinase-producer at another site.

Finally, rabbits were immunized with penicillinase to counteract the penicillin-destroying action of the penicillinase-producing bacteria.

RESULTS

In Paper I experimental wounds were treated with penicillin vaseline, the infection being due to penicillin-sensitive *pasteurella,* or penicillin-sensitive *streptococci,* or one of these strains in combination with penicillin-sensitive *staphylococci* or *staphylococci* producing extracellular penicillinase.

Macroscopically the *pasteurella*-infected wounds appeared open, moderately suppurating, and with pronounced inflammatory oedema. The edges were necrotic, and surrounded by a bluish-violet area. The wounds infected with β-*streptococci* were surrounded by slight oedema, the edges being reddish. The *staphylococcal* infections showed only slight inflammation. After penicillin treatment, the wounds healed and were covered by a crust within a few days.

The results show that the rabbits with a simple infection due to *pasteurella* were cured by penicillin vaseline in a very short time. They soon regained normal temperature, were in good condition, and showed a moderate gain in weight. The untreated animals, on the other hand, died in about 5 days.

Wound infections due to penicillin-sensitive staphylococci *only,* or to *staphylococci* producing extracellular penicillinase, caused no weight-loss or rise in temperature.

In the control experiments, where a sufficient quantity of penicillinase was added to penicillin vaseline, the therapeutic effect of the penicillin was abolished. In these experiments both the treated and untreated rabbits survived for an average of about 5 days.

Penicillin vaseline had a good therapeutic effect in mixed infections due to penicillin-sensitve *pasteurella* and penicillin-sensitive *staphylococci* at the same site of infection. On the other hand, penicillin vaseline had no therapeutic effect on mixed infections due to penicillin-sensitive *pasteurella* together with non-pathogenic *staphylococci* producing extracellular penicillinase.

Similar results were achieved in experiments with penicillin-sensitive *streptococci* instead of penicillin-sensitive *pasteurella.*

Rabbits infected with only *streptococci* were cured very quickly by penicillin vaseline, but untreated rabbits with the same infection showed pronounced rise in temperature and loss of weight, although they did not die during the observation period.

Penicillin vaseline was also satisfactory in the treatment of mixed infections due to penicillin-sensitive *streptococci* and penicillin-sensitive

staphylococci, but it had less effect when the *streptococci* were present together with *staphylococci* producing extracellular penicillinase.

This state of affairs can probably be explained by the fact that the *streptococci* were less pathogenic than the *pasteurella*; and when that they occurred together with *staphylococci* producing extracellular penicillinase at a single site of infection they developed less readily in the wounds, so that less penicillinase was produced than when *pasteurella* was present.

In some of the series rabbits were treated with procaine penicillin intramuscularly instead of penicillin vaseline locally, and the results were very much the same.

In Paper II some other pencillinase-producing bacteria were examined under the same experimental conditions as in Paper I. The bacterial strains chosen were *Escherichia coli*, producing much extracellular penicillinase, *Pseudomonas pyocyanea*, a weak producer of extracellular penicillinase, and strains of these two species producing intracellular penicillinase.

Wound infections due to *Escherichia coli* and *Pseudomonas pyocyanea* were not evident to the naked eye, but sometimes a slight redness of the wound edges could be seen.

In Paper I the technique of assaying the production of extracellular penicillinase of a strain has been described. Paper II contains a list of the strains of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas pyocyanea* producing intracellular penicillinase. These strains are relatively penicillin-resistant, but by the cup method it could not be demonstrated that they produced exracellular penicillinase. If, however, they were allowed to grow in broth containing moderate concentrations of penicillin for 48 hours, after which time most of the bacteria had been autolysed, and then removed by centrifuging, the occurrence of penicillinase could be demonstrated by the cup method. The growth of the strains used in the experiments was inhibited by the following dilutions of penicillin in broth: *Staphylococcus aureus*, 8 units/ml; *Escherichia coli*, 4 units/ml; and *Pseudomonas pyocyanea*, 2 units/ml.

The results showed that neither intra- nor extracellular varieties of *coli* and *pyocyaneus* bacteria, alone or together, in any way affected the general condition of the rabbits. Thus both the treated and untreated animals behaved alike in all respects.

In mixed infections, where penicillin-sensitive *pasteurella* or *streptococcus* bacteria existed together with *coli* or *pyocyaneus* bacteria producing intracellular penicillinase no inhibition occurred when the animals were treated with procaine penicillin.

The *coli* bacteria producing much extracellular penicillinase had the same effect on the penicillin as the penicillinase producing *staphylococci*. In mixed infections the *pyocyaneus* bacteria, which produced less extracellular penicillinase than the *staphylococci* and *E. coli*, caused some inhibition of the penicillin, but the drug was not wholly inactivated. Thus all the untreated, compared with only 6 of the 10 treated, animals died.

In one series Paper II attempts were made to eliminate the peni-

cillinase activity in mixed infections by increasing the dose of penicillin. The experiments were arranged analogously with previous series in Paper I and II, *staphylococci* (large amounts of extracellular penicillinase) and *pseudomonas* (weak producer of extracellular penicillinase) again being used, but with a dose of 90.000 units of penicillin instead of 30.000 units. A control experiment, with no infection, showed that the rabbits were not affected by this high dose of procaine penicillin, which had a somewhat better therapeutic effect than the small dose in the *staphylococcus* experiment; but the extracellular penicillinase produced still caused pronounced inhibition of the therapeutic effect. 8 of 10 treated and all 10 untreated animals died.

Similarly, in the *pseudomonas* experiment the large dose of procaine penicillin had a somewhat better therapeutic effect on penicillin-sensitive bacteria than the small dose.

In Paper III, two isolated sites of infection were studied, one being infected with non-pathogenic penicillinase producers, and the other with penicillin-sensitive pathogens. These investigations were made to establish whether a spread of penicillinase can take place from a local site of infection to the whole organism, and, if such is the case, whether the occurrence of penicillinase in the organism inhibits the therapeutic effect of penicillin on penicillin-sensitive bacteria elsewhere in the body.

β-*streptococci*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas pyocyanea* were injected intracutaneously. After injecting β-*streptococci*, redness and infiltration could be observed during the observation period within an area of one sq. cm. The other bacteria produced inflammation similar to that caused by *streptococci*.

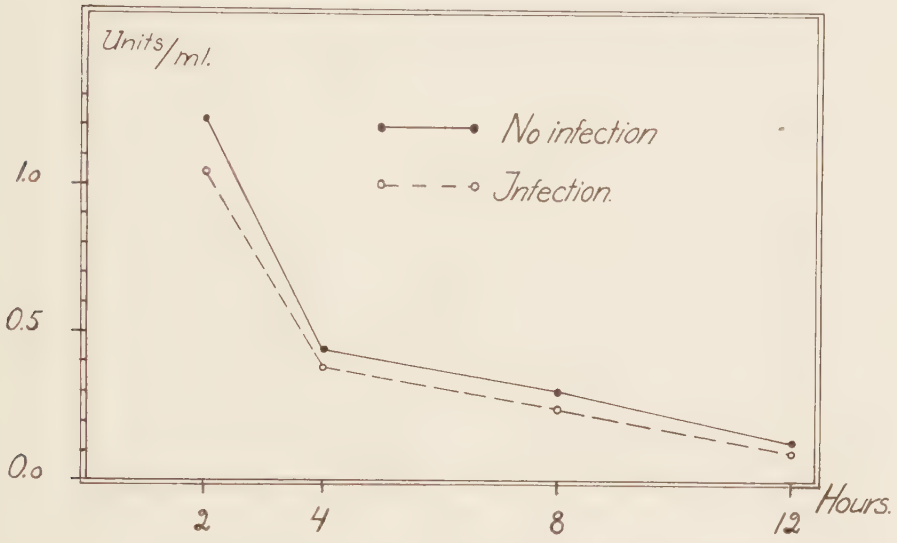
On penicillin treatment the redness and infiltration caused by intracutaneous injection of β-*streptococci* disappeared in a few days.

The experiments showed that procaine penicillin had a therapeutic effect on penicillin-sensitive *pasteurella* infections occurring simultaneously with infection at another site due to *pyocyanei* or *staphylococci* both of which produce extracellular penicillinase).

Experiments with *E. coli*, a strong producer of extracellular penicillinase, gave in contrast different and inconclusive results. Even the control experiments with only *coli* bacteria, were difficult to evaluate: 9 of the 10 penicillin-treated rabbits died, and all the untreated animals survived. When the experiments were repeated, however, all the test animals survived. Some other similar experiments are described in an earlier paper (83), which is, however, not included in this thesis as the investigations are not complete.

In animals with two sites of infection, *pasteurella* at one and *coli* bacteria producing extracellular penicillinase at the other, 9 of the 10 penicillin-treated and all the untreated animals died; but when the experiments were repeated all the treated animals survived. The rabbits were nevertheless somewhat more affected by the double infection. The results are difficult to interpret, and no conclusions can be drawn.

In connexion with these experiments the concentrations of penicillin was estimated in 20 2-kg rabbits, arranged in two groups of 10, which had received 30,000 units of procaine penicillin intramuscularly. One group had a local site of infection due to *staphylococci* producing extracellular penicillinase. The concentrations are given in the figure. The difference between the average concentrations in the sera of the two groups is not statistically significant



Penicillin concentration in serum after injecting 30,000 units of penicillin intramuscularly. Each value represents the mean for 10 rabbits.

The survial of the penicillin treated animals of Paper III was probably due to the persistence of a serum-penicillin level sufficently high to affect a *pasteurella* or *streptococcal* infection, despite the presence elsewhere in the body of a lesion infected with organisms producing extracellular penicillinase. In the investigations of Papers I and II, where penicillin had no effect at the common site of infection, penicillin had clearly been destroyed to such an extent that it no longer influenced *pasteurella*.

A series of experiments was performed on 30 rabbits, using commercial penicillinase.

In all the rabbits experimental lesions were infected with *pasteurella* bacteria.

The series was arranged in 3 groups of 10 rabbits each.

(a) The rabbits were treated with procaine penicillin, and all survived.

(b) Penicillinase was injected intravenously in a dose of 2000 units per day for 10 days, and the animals were treated with procaine penicillin. All the animals died.

(c) Controls. No treatment was given, and all the animals died.

It is clear from the results that intravenous administration of penicillinase interferes with the therapeutic effect of penicillin upon penicillin-sensitive *pasteurella*.

In Paper IV the rabbits were immunized with penicillinase to establish whether the antipenicillinase that had been produced during the process of immunization could neutralize the extracellular penicillinase produced by the infecting bacteria.

Sera of immunized rabbits were also tested in vitro against precipitating and protective antibodies.

Precipitation

In 20 sera from the immunized rabbits precipitation of antipenicillinase could be demonstrated in serum dilutions 1: 64—1: 256. Normal rabbit serum gave no precipitation.

Protective action in vitro

It was shown that 0.25 ml of serum containing antipenicillinase protected 4000 units of penicillin against inactivation by 100 units of penicillinase. In control experiments using normal rabbit serum no protection could be achieved.

Protective action in vivo

Sixteen days after the final dose of penicillinase, penicillin-sensitive *pasteurella* together with *staphylococci* producing extracellular penicillinase were introduced into experimental skin lesions. Treatment with procaine penicillin was then commenced. The effect was good, possibly owing to inhibition of the penicillinase by antipenicillinase in the circulating blood.

The mortality in the group treated with penicillin and immunized with penicillinase was 35 %, and in that treated with penicillin but not immunized 100 %. The difference is highly significant ($P < 0.001^{***}$).

GENERAL DISCUSSION

Theoretically penicillinase may inactivate penicillin either in the circulating blood or at the site of infection.

Commercial penicillinase administered intravenously has the same effect upon penicillin as has the penicillinase produced by the organisms used in these experiments, though it is again not clear whether inactivation takes place in the blood or at the site of infection. If the latter is the case penicillinase must pass out from the blood into the infected tissue.

The results of the experiments in which two separate lesions were present are not easily evaluated. One interpretation would be that penicillinase is produced at one site and transported via the blood stream to the other site of infection, although the concentration of penicillinase in the blood is too low to influence the therapeutic effect of penicillin. Another possibility is that penicillinase remains at the site of production. No clear-cut conclusions may be drawn from the findings of these experiments, though it is clear that the penicillinase had much greater effect when it was produced locally than when it had to be transported from a distant site.

It is also evident that immunity to penicillinase can be induced. Concerning the precipitation which took place when commercial penicillinase was added to antipenicillinase, it is not clear whether the phenomenon was due to a specific antigen — antibody reaction or whether it could have depended on some other substance or substances specific for the organism, B.cereus, used in the manufacture of the enzyme. Similarly it is not obvious whether the protection of the penicillin against penicillinase in vivo was due to the presence of a non-specific link in the enzyme system.

The protective action of antipenicillinase-immune serum on penicillin is of particular interest in view of the apparent increase in pathogenicity of certain penicillinase-producing *staphylococci* on administration of penicillin (83). Theoretically it would of course be possible to tackle the problem of penicillin-resistant *staphylococci* in hospital infections, for example, by immunizing patients against penicillinase, thereby making the infecting organisms accessible to penicillin therapy.

The clinical importance of the phenomenon of penicillinase-immunization cannot be evaluated before experiments have been carried out on animals, and later on human subjects, to establish the length of time required to form antipenicillinase.

CONCLUSIONS

The following conclusions are drawn from the results of experiments carried out on rabbits to test the effect of penicillin on penicillin-sensitive pathogenic bacteria in the presence of non-pathogens producing extracellular penicillinase.

1. The presence of non-pathogenic bacteria producing extracellular penicillinase inhibits the antibiotic action of penicillin. This effect is most obvious with *staphylococci* and *coli* bacteria, which are strong producers of extracellular penicillinase, and is less evident when penicillinase production is weaker (e.g. with *pseudomonas*).

2. The therapeutic effect of penicillin vaseline and procaine penicillin was likewise impaired by the addition of commercial penicillinase. The same effect is achieved by intravenous injection of commercial penicillinase.

3. The presence of penicillinase-producing organisms at a site apart from the site of infection had little if any effect on the antibiotic action of penicillin.

4. The presence of antipenicillinase in the serum counteracted the effect of penicillinase on penicillin.

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